Final Report For
United Sorghum Checkoff Program (USCP)

USCP Contract Number: HVM10-15

Project Title: Expanding utilization and increasing value of sorghum fractions via microbial conversion

Institution or Organization: Prairie AquaTech

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EXECUTIVE SUMMARY

This project was designed to create value added for sorghum fractions such as sorghum mill feed (SMF) and sorghum hominy (SH) and increase their demand and profitability. While the relatively low protein, high fiber, and anti-nutritional content of SMF and SH limits their inclusion in traditional livestock feeding applications and higher value aquafeeds, other targeted aquafeeds uses particularly with lower trophic fish such as tilapia and catfish remain likely possibilities. Aquafeeds production for these two species on a global basis exceed two million metric tons and require feed ingredients that are priced competitively to enable them to displace existing plant based sources such as soy, wheat and wheat middlings (mids). Thus, the project's specific objectives include: 1) Optimize, scale-up, and validate the microbial conversion process for SMF/SH, 2) Evaluate both bioprocessed SMF/SH and/or SH as possible feed ingredients for tilapia aquaculture and 3) Estimate commercial scale production costs (CAPEX/OPEX), market size and value, return on investment (ROI) potential, and timeline for deployment.

Our results summarized in this final report show that SH can effectively be utilized as a feedstock for aerobic fermentation using the organism, *T. reesei,* to increase the protein content and desirable amino acid composition of SH. The resulting bioprocessed SH product can be used effectively as a feed ingredient in extruded aquafeeds at inclusion rates >20% to create high quality floating pelleted aquafeeds. Tank-based feeding and growth trials using tilapia were conducted to evaluate both SH and bioprocessed SH performance as feed ingredients. These data reveal that SH can be used at inclusion rates of 25% of custom formulated diets and achieve fish performance that is similar to commercial tilapia feeds. By contrast, while tilapia fed pelleted feeds containing SH bioprocessed with *T. reesei* display similar growth, their feed efficiency is reduced as compared to control tilapia and the resulting fillets from market size fish contain an undesirable off-flavor that is highly undesirable. Taken together, use of *T. reesei* to bioprocess SH as a feed ingredient for tilapia aquaculture appears to be problematic.

By contrast, SH can be combined successfully with another high value bioprocessed feed ingredient called ME-PRO® that is produced using aerobic fermentation of washed soybean meal to create both high quality extruded pelleted aquafeeds as well as utilize a very low cost screw press methodology (<$15,000 CAPEX investment) to create acceptable aquafeeds that perform well in tank-based feed trials. Taken together, these data support further efforts to focus on development of SH as a replacement for the combination of whole wheat and wheat midds that are widely used by the aquafeeds manufacturing sector as well as possible creation of new markets for SH as a key feed ingredient to allow fish farmers in isolated or underserved areas of the globe to manufacture suitable aquafeeds without the large (>$1,000,000 USD CAPEX) investment to establish an extruded feed mill in their region.

TECHNICAL OBJECTIVES

The **overall goal** of the project is to convert or utilize various sorghum fractions as novel ingredients in aquafeeds. One approach is to convert sorghum fractions into highly digestible, high protein feeds to replace other feed ingredients like fish meal in aquaculture. Another approach is to utilize existing sorghum fractions in novel ways to create high quality aquafeeds that contain sorghum rather than other plant-based products that are currently being utilized by the aquafeeds manufacturing industry.

The **specific objectives** of the project include**:**

- Optimize, scale-up, and validate the microbial conversion process for Sorghum mill feed (SMF/Sorghum hominy (SH) to maximize protein levels and digestibility, while minimizing processing costs
- Evaluate the bioprocessed SMF/SH as a feed ingredient in selected aquaculture diets, while determining their relative value in comparison to other feeds
- Explore whether existing sorghum products (SH) could be utilized in novel ways to replace other carbohydrate based feed ingredients such as wheat so as to create increased demand for SH products.
- Estimate commercial scale production costs (CAPEX/OPEX), market size and value, return on investment (ROI) potential, and timeline for deployment for a bioprocessed SMF/SH product or utilization of SH in the aquafeeds industry by present day feed mills.

BACKGROUND

Profitability of the sorghum industry is limited by the composition and relatively low value of sorghum fractions such as sorghum mill feed (SMF) and sorghum hominy (SH). While sorghum itself has been successfully utilized as feedstock for the ethanol industry similar to that of corn, this process has generated sorghum DDGS which can also be utilized as distillation by-product solids in a variety of animal feed uses. In this regard, previous studies by Lochmann and colleagues (1) using channel catfish showed that sorghum distillers grains with solubles (S-DDGs) could be effectively utilized by pond-reared catfish at inclusions rates of 20% with good growth and feed conversion as compared to either corn-based DDGs or soy bean meal used as a control. Since S-DDGs have a lower carotenoid content vs. corn DDGs, it is possible to use a mix for white fleshed fish to prevent undesirable fillet coloration at high inclusion rates in catfish feeds. Although such pilot scale studies are promising and show the possible use of sorghum products as aquafeeds ingredients, price competition for such products already exists in foreign markets. As a result, we have focused on the need to define the uses of sorghum in other fish species such as tilapia or rainbow trout as well as whether fermentation with other microbial species other than yeast might provide for still further value added.

Previous studies that have evaluated the use of sorghum products in tilapia have yielded some promising but not conclusive results. Early studies by Furuya et al. (2) and Al-Ogaily et al. (3) using non-extruded diets showed that low but not high-tannin sorghum meal could be substituted for corn meal in tilapia diets. More recent studies (4, 5) have demonstrated that sorghum starch can be used as a feed ingredient at inclusion rates up to 30% in juvenile tilapia as a source of dietary carbohydrate. However, affordable (~$600/ton) feed grade sorghum starch products often have higher tannin contents which reduce their value as aquafeeds ingredients since high tannin sorghum products reduce tilapia growth rates.

It is well known that the relatively low protein, high fiber, and anti-nutritional content of SMF and SH limits their performance in traditional livestock feeding applications, and precludes the use of these products at significant inclusion rates (>15%) in higher value aquafeeds such as those utilized for production of salmonids. Thus, careful studies of other fish such as tilapia that are more capable of using plant-based carbohydrate sources at high inclusion levels are the focus of this effort. A central goal of this project is to determine whether SMF and/or SH can be bioprocessed using appropriate microbial enhancement to produce a product suitable for inclusion in aquafeeds at high (>25%) inclusion rates. It is envisioned that microbial bioprocessing of sorghum will increase both the protein content and increase the digestibility of SH using a process that will be commercially viable after appropriate scale-up. In this regard, we have focused on development of a microbially-enhanced process that would provide a sorghum product suitable for use in aquafeeds in key fish species that would either be sold at prices below that of commodity-based soybean meals for tilapia or, alternatively, a higher priced product that would compete with soy protein concentrates (SPCs) at a price range of less than $1000/ton.

In addition, the carbohydrate content of SH might be utilized for its mechano-elastic properties in extruded aquafeeds manufacturing so as to stabilize pellet integrity and produce a high quality floating pellet. Currently, extrusion based feed mills utilize starch from wheat and wheat middlings as an inexpensive source of starch to gelatinize in the extrusion process to produce high quality pellets. A possible new use and expanded market for SH carbohydrates in both extruded and non-extruded aquafeeds would be their possible use as an affordable carbohydrate source in aquafeeds formulations. Lastly, lower trophic fish such as tilapia and catfish are farmed in many areas throughout North America and the world that either are distant from high quality feed mills but are located in areas in close proximity to significant sorghum production. A possible very low-cost method using SH to produce acceptable feed for tilapia production could be benefit to make smaller more isolated tilapia farmers more cost competitive with larger more technologically dependent aquaculture producers.

In previous work with tilapia where we have tested other plant-based feedstocks after their microbial enhancement with different microorganisms, we have discovered that the complete pilot scale testing of such candidate feed ingredients must include taste testing by a panel of 4-6 persons (conducted in a blinded manner where identity of fish fillets is known only to the taste panel coordinators). This commercial taste testing of the resulting fillet product in our studies is important since different microorganisms impart different trace flavors in the resulting fillets and this will be performed in addition to traditional performance evaluation metrics (growth, survival, FCR, etc.) in fish fed various microbially-enhanced feed ingredients to compare with our prototype sorghum-based feed ingredients as summarized below.

RESULTS

OBJECTIVE 1: Optimize, scale-up, and validate the microbial conversion process for SMF/SH to maximize protein levels and digestibility, while minimizing processing costs

Evaluation of Possible Fermentation Improvements Using Enzymatic Saccharification and Other Pretreatment Methods.

Previously, we reported that cellobiose was the major sugar present in sorghum hominy after mixing the sorghum in a liquid slurry and heating the liquid to 50°C for 24 hours. Moreover, we observed that SH slurry glucose concentrations were highest following saccharification with the commercially available cellulose enzymes Ronozyme VP and Viscozyme L. In follow up studies, we sought to optimize any pre-treatment steps using an enzyme cocktail for hydrolyzing SH. In this regard, we conducted multiple saccharification trials using a total of 6 different commercially available enzymes. These data show that Ronozyme VP followed by Viscozyme L were the best enzymes tested and resulted in the best sugar profile for subsequent possible aerobic fermentation. The starch content of SH was measured as 4.13% (dry basis) according to the Megazyme protocol. We then evaluated the performance of SH after pre-treatment with commercial starch degrading enzymes (alpha amylase and amyloglucosidase, AMG) in SH saccharification trials. However, these trials yielded minimal results. In addition, various other pretreatment methods (Kady mill, hot water cook, dilute alkali, and dilute acid methods) were evaluated in an effort to determine a possible pretreatment protocol for SH prior to aerobic fermentation with a selected organism. **The results of these data were reported previously in interim reports and are not summarized in detail here. Moreover, scale up efforts described below that resulted in bioprocessed SH product that was tested in fish trials did not utilized any pre-treatment step prior to aerobic fermentation**.

<u>Strain Selection and Shake Flask Testing</u>

Previously in 250 mL shake flask testing, we found that *R. oligosporus, T. reesei, A. pullulans 23,* and *N. crassa* grew very well on the sorghum hominy feedstock under submerged culture conditions and these organisms increased the protein content of the resulting solids after fermentation. Table 1 shows the final protein contents for the solids produced from SH fermentation with various organisms. Note that *T. reesei* and *R. oligosporus* yielded the highest protein concentration yield while *A. pullulans* and *P. kudriavzevii* produced fermented solids with a protein content of approximately one-half that produced by the leading organism candidates. It is noteworthy that while *A. pullulans* performed poorly in production of high protein in the separated solids, but as the project progressed, it was it was noted to significantly reduce the viscosity of the fermentation broth which is a challenge to accomplish with *T. reesei* fermentation conditions where appropriate mixing and commercial scale up will be essential as discussed below. Therefore, further experiments were conducted on *A. pullulans* despite its poor performance to enhance protein content of SH. Lastly, note that fermentation of SH by some organisms (*T. reesei*) cause a reduction in pH as compared to others like *N. crassa* or *P. kudriavzevii* which increased fermentation broth pH.

Table 1: **Summary of fermentation broth pH and crude protein values of the final solids from flask trials. See text for details.**

Organism	Start pH	Average End pH	Average % Protein after 5 day growth period	Protein % gained compared to control
Un-inoculated Control	5.66	5.32±0.15	13.26±0.18%	0.00%
A. pullulans 23	3.00	3.52±0.13	16.67±0.31%	25.77%
F. venenatum	5.60	5.39±0.08	19.43±0.43%	46.54%
T. reesei	5.60	4.11±0.12	20.11±1.02%	51.64%
P. variotii	5.60	4.56±0.05	17.86±0.06%	34.74%
N. crassa	5.60	6.21±0.05	18.30±0.24%	38.02%
R. oligosporus	5.60	5.45±0.06	21.26±0.27%	60.38%
P. kudriavzevii	3.00	3.49±0.11	16.70±0.46%	25.98%
M. circinelloides	5.60	5.28±0.28	17.27±0.36%	30.26%

After review of these data summarized in Table I, we assessed the performance of four choice organisms (*T. reesei, A. pullulans, R. oligosporus and N. crassa*) using 7-day trial flask incubations under submerged fermentation conditions at solids loading rates of 10% where we obtained daily samples of incubation slurry for the purpose of mass balance and protein analyses**. These results indicated *T. reesei* was the best performing fungus as it yielded a value for fermented solids protein concentration of ~ 20% was reached within 48 hr. (Figure 1).** By contrast, protein concentration yields for *A. pullulans*, *R. oligosporus* or *N. crassa,* were either of smaller magnitude *(A. pullulans*) or required approximately twice the interval of time (100-140 hr.) to achieve a similar increase in solids protein content (*R. oligosporus* & *N. crassa*). As discussed previously, more rapid microbial bioprocessing is preferred to enable commercial production cycles of plant batch production of product to occur at a more rapid rate (faster plant throughput).

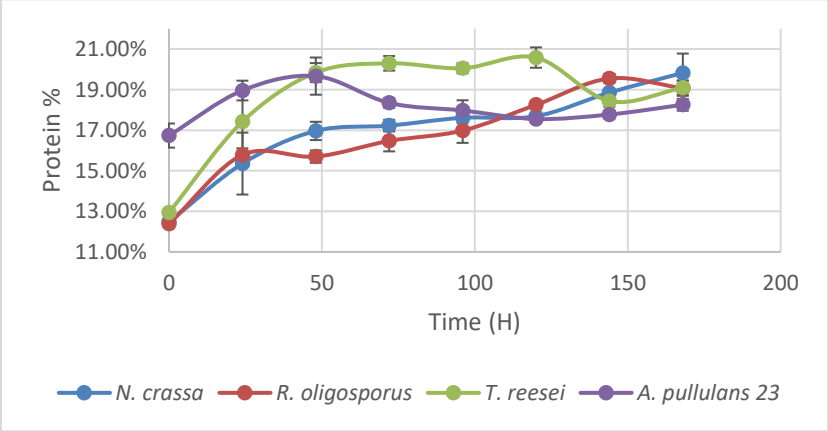

Figure 1. Comparison of different protein contents of fermented solids produced by four microorganisms screened previously using flask cultures over a 7 day incubation interval. Note that the starting protein concentration for *A. pullulans* is ~17% as compared to ~13% for all others. See text for details. Error bars denote standard deviation.

These same extended flask trials lasting a total of 170 hours (see Figure 1) were also used to obtain mass balance information on these same organisms. These data revealed that *A. pullulans 23* yielded a final protein biomass of ~ 8 grams while *T. reesei* and *R. oligosporus* each yielded protein biomass yields of ~ 7 grams respectively while *N. crassa* yielded only ~6 grams of protein.

Analyses were also performed on the supernatants of samples shown in Figure 1 to determine both their total solids (Figure 2) and protein contents (Figure 3). Note that supernatant solids decreased gradually over 170 hr. studied in all fungi to yield a final value of 2-3%. Of note is that *T. reesei* displayed the lowest initial supernatant solids fraction that was reduced the least during the fermentation interval. By contrast, *A. pullulans* yielded the highest initial supernatant solids that decreased significantly over the same interval to approximately the same value as *T. reesei*. The supernatant composition (Figure 2) after the 7 day incubation of the treated SH was relatively consistent between the treatments analyzed with 2.0-2.5% of the supernatant being solids.

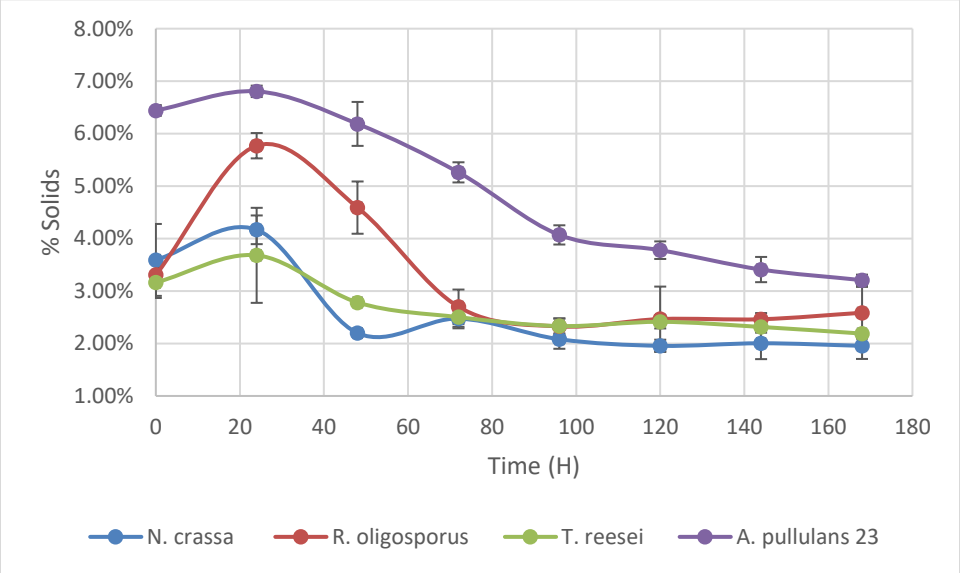

Figure 2. Comparison of total solids (dissolved and suspended) displayed by each of 4 different microorganisms grown on SH in flask cultures. See Figure 1 and text for details. Error bars denote standard deviation.

The supernatant protein content of SH fermented using *A. pullulans 23* remained low (~4%), while the protein content of all other supernatants increased significantly to achieve greater than 10% (Figure 3). Lastly, the pH of the fermentation broth was also monitored for each of the 4 organisms studied (Figure 4). As expected from data shown in Table I, the pH for *T. reesei* decreased over the interval studied.

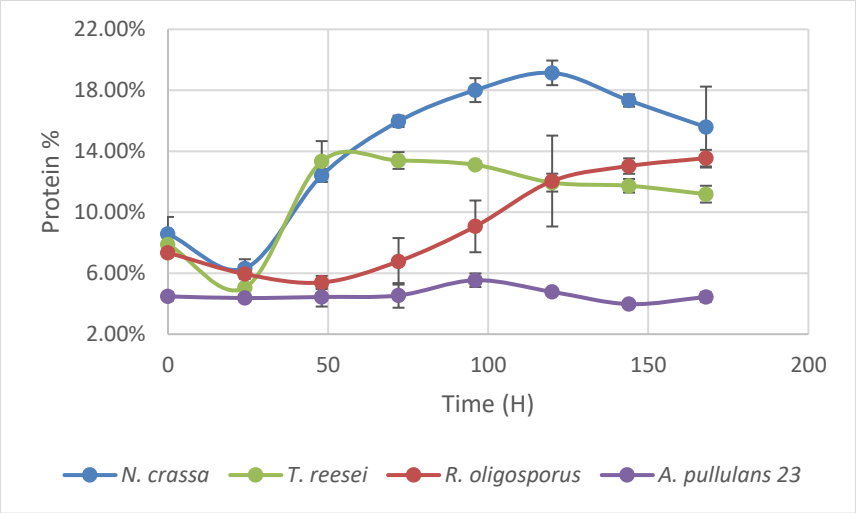

Figure 3. Comparison of total protein content of supernatants from samples of 4 different microorganism fermentations as described in Figures 1 &2. See text for details. Error bars denote standard deviation.

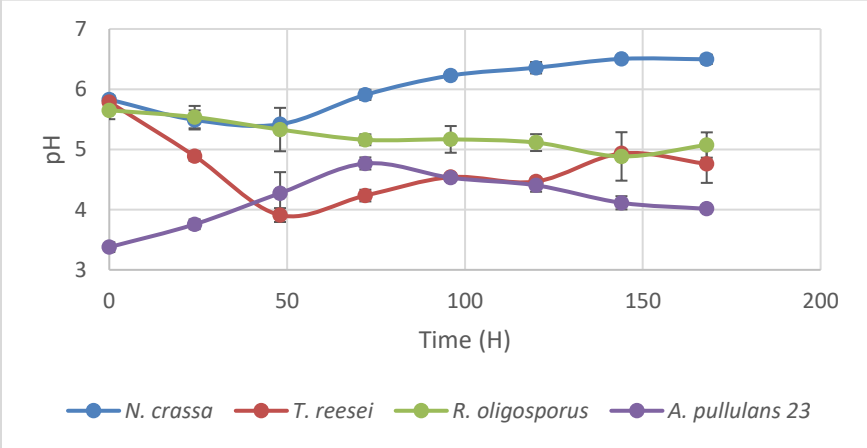

Figure 4. Comparison of changes in the pH of broth produced by fermentation of SH using one of four different microorganisms. See Figures 1-3 and text for details. Error bars denote standard deviation.

In summary, on the basis of these data in Figures 1-4, fermentation of SH using *T. reesei* in flask cultures appeared to be the best choice for scale up in larger fermentation bioreactors. This selection is made on the basis of a significant increase in the protein content of fermented solids by 72 hr. together with only a 2.5-3.0% total suspended solids fraction possessing ~14% protein. Thus, scale up of *T. reesei* fermentation was performed as described below.

Bioflow 5 Liter Scale Up Testing

Bioflo experiments (5 L total volume) were then conducted in triplicate using *T. reesei* as the best performing organism. As a control for comparison of other bioflo parameters with those generated using *T. reesei,* identical experiments were conducted using *A. pullulans* since our group has significant experience with such fermentation scale up parameters using other feedstocks like soybean meal. The SH medium was prepared at a 10% solids loading rate and autoclaved prior to cooling and setting system parameters (Temp=30^{o}C, Agitation=250rpm, Aeration=1.0v/v/m, pH=5.6 for *T. reesei* and 3.0 for *A. pullulans*. Each bioflo was then inoculated with 1% v/v

inoculum prepared by growing 100mL GYE for 24 hr. Daily samples (100mL) were taken via pipette, checked for purity via gram stain and streak plate, then analyzed for mass balance and protein yields.

We observed significant growth of each organism in all 6 fermentation runs. As shown in Figure 5, *A. pullulans* displayed a significantly higher total solids and total suspended solids yield as compared to *T. reesei*. The fermentation with *A. pullulans* actually resulted in a 10% increase in total solids (starting ratio of loading is 10% solids loading rate) and 21% increase in total suspended solids. However, these characteristics are of limited utility due to this organism's limited ability to increase sorghum's protein content. By contrast, *T. reesei* decreased total solids by 66% and total suspended solids by 16% while increasing their protein contents by more than 65% and 100% respectively. Of note is the large amount of suspended filamentous *T. reesei* cell mass that developed during the fermentation such that the SH feedstock was covered with a thick matt of mycelia (Figure 6). As a result of this growth, mixing of the SH feedstock and growing *T. reesei* mycelia was impaired under standard benchtop bioflo conditions. This resulted in the very low solids content in the liquid fraction of the fermenters as displayed in figure 5.

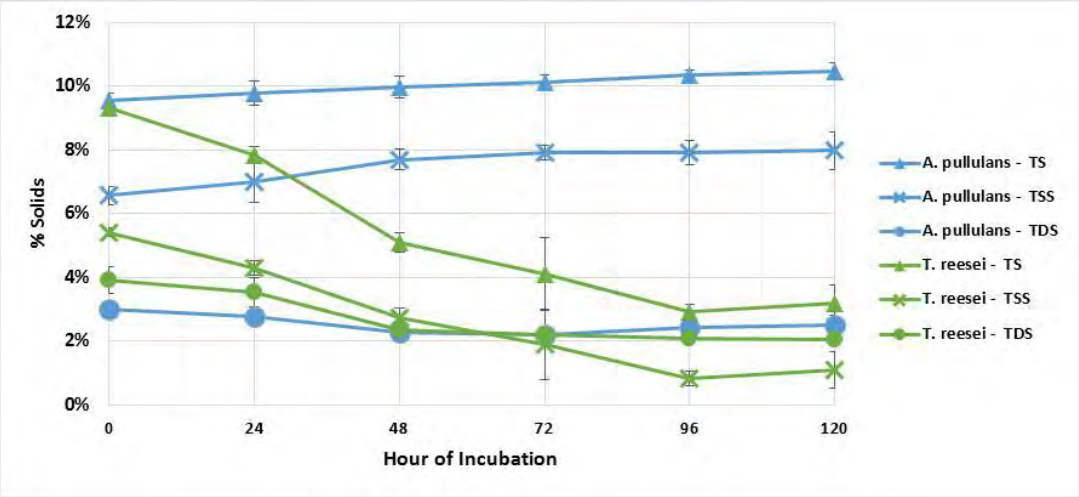

Figure 5. Comparison of daily mass balance (TS=total solids, TSS=total suspended solids, TDS=total dissolved solids) for *A. pullulans* and *T. reesei* grown on sorghum hominy in 5L fermenters for 5 days. Error bars indicate standard deviation.

Upon harvesting bioflo fermenters after 72 hr., we observed that their contents are very heterogeneous as the bulk of the partially fermented SH feedstock was sticking either to the bottom of the fermenter or its impeller shaft, or, alternatively, to in other areas away from the harvesting port (Figure 6). The presence of this heterogeneity in fermentation slurry composed of SH and *T. reesei* provided significant challenges to obtain accurate sampling data from the 5 liter bioflo apparatus as compared to samples obtained from shake flasks.

Slurry pH throughout bioflo fermentation yielded a trend almost identical to previous experiments in shake flask cultures (Figure 7). The pH of *T. reesei* broth consistently drops sharply until 48 hours when it reaches pH=4 and then gradually rises to about 4.7 at 5 days. The *A. pullulans* broth starts at a much lower pH, then consistently increases to pH=4.5 at 72 hours and then decreases the remainder.

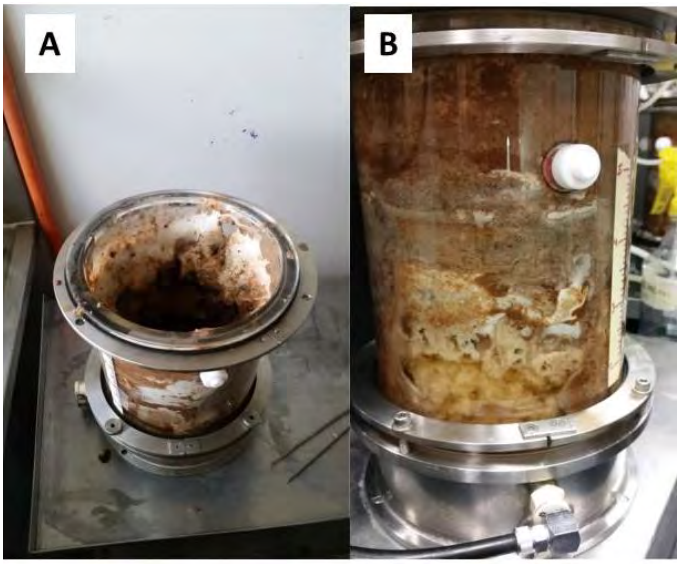

Figure 6. Representative photographs of 5 liter Bioflo apparatus after an interval of ~60-72 hr. of fermentation of SH with *T. reesei.* As shown in Panel A (left), the SH feedstock and *T. reesei* filaments stick to the slides of the fermenter. As shown in Panel B (right), the viscous nature of the fermentation slurry resulted in an apparent heterogenous mixture of products which complicates interpretations of samples obtained via the sampling port. See text for details.

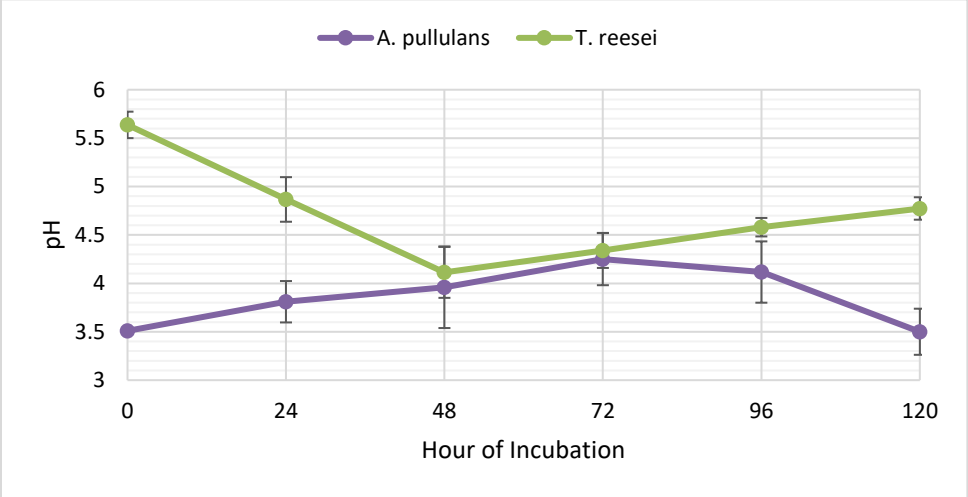

Figure 7. Comparison of daily pH of slurry during 5L fermentation with A. pullulans and *T. reesei*. Error bars indicate standard deviation.

Analysis of protein concentrations under 5 liter bioflo conditions (Figure 8) revealed results consistent with data obtained in shake flask cultures (see Figures 1-4). The final solids protein content of *T. reesei* fermented SH averaged 23±4%, a relative increase of 62% as compared to an initial SH protein content of 14%. By contrast, the final solids protein content of *A. pullulans* fermented SH was 17±1% or a 25% increase as compared to the initial SH protein content. Note the large variability present in the protein content of *T. reesei* fermented SH replicates which is attributable to the heterogeneous nature of the *T. reesei*-SH biomass growing under these 5 liter standard bioflo conditions as described above (see Figure 6).

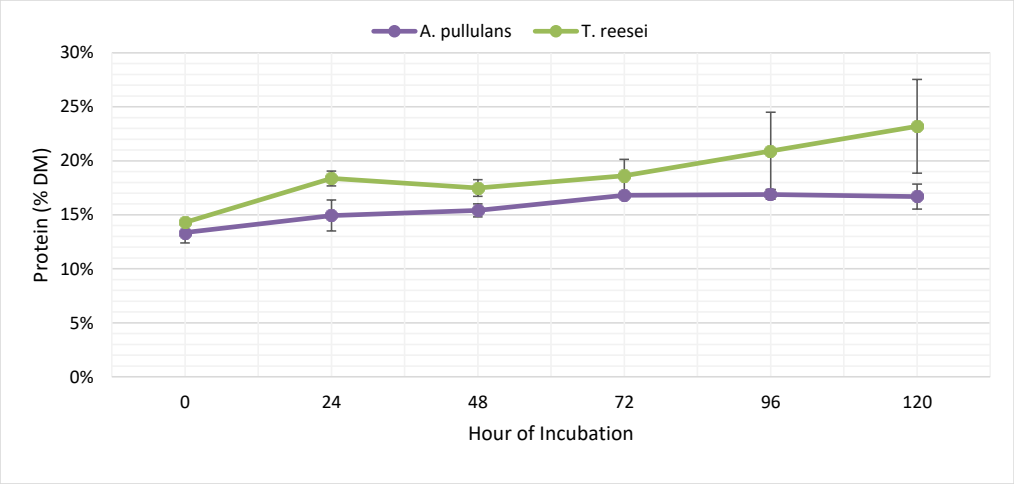

Figure 8. Daily solids protein over a 5-day incubation of *A. pullulans* and *T. reesei* on SH feedstock in 5 L bioflo fermenters. See text for details. Error bars denote standard deviation.

As described in Figure 6, we encountered difficulties in achieving continuous adequate mixing of the SH-*T. reesei* biomass during fermentation under submerged liquid conditions using standard bioflo formats. As an alternative, we performed a pilot scale experiment where SH was fermented under solid state conditions for identical intervals of time. As shown in Figure 9, these results are promising in that a nearly identical increase in protein content of the fermented SH-*T. reesei* biomass was achieved without the requirement of holding liquid fermentation cultures. Overall yield of starting SH feedstock was ~67%. If proven to be similar after scale up, this solid state fermentation format for production of SH product could significantly reduce the capex requirements for industrial scale SH-*T. reesei* product production (see Objective 3 below).

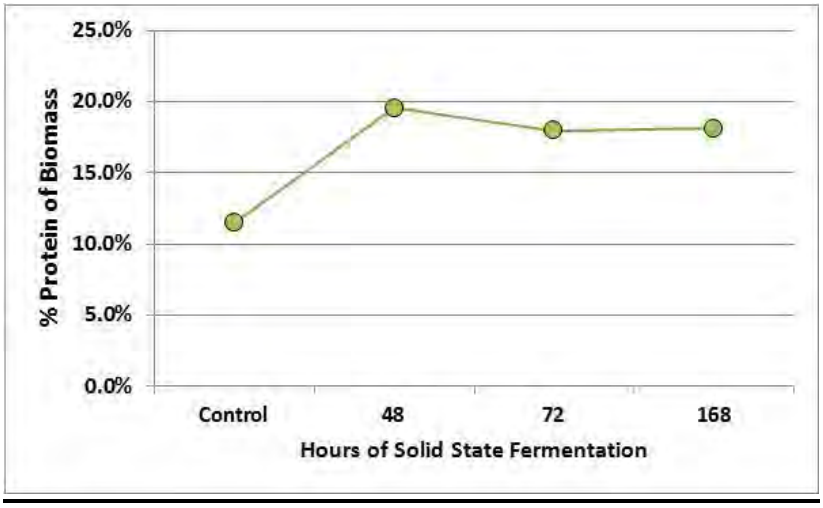

Figure 9: Protein content of sorghum hominy after fermentation with *T. reesei* under solid state conditions for various intervals. See text for details.

Trials Using 150L Scale-up Mode

In order to demonstrate initial scale-up performance under submerged liquid fermentation conditions, a pilot-scale trial was completed using a 150 L fermenter, and conditions listed in Table

2. After steam sterilization of the fermenter for 60 minutes, the fermenting biomass was inspected daily for possible contamination using streak plates and gram staining. No contamination was observed throughout the 72 hour fermentation interval. Good growth was visually observed and was similar to other trials using *T. reesei* on SH, where a fluffy / lightweight "cake" of mycelia was produced. The pH values observed during incubation were consistent with previous trials (Figure 10). SH-*T. reesei* solids protein content increased by over 85% (i.e. 11.5% (db) for SH feedstock to 21.3% (db) final product) as shown in Figure 11. The 72 hour final material was separated using one of two different centrifugation techniques. In the first method where a laboratory centrifuge (4,000 rpm for 5 min) was used, the resulting pelleted solids displayed as protein content of 17.8% (db). This value was lower as compared to processing of the same SH-*T. reesei* biomass with the second method involving a decanter centrifuge (20.1% db). After obtaining these data, most of the final SH-*T. reesei* product was separated via decanter centrifuge and then subjected to 2 sequential washing processes using fresh water. This process further increased its protein content to 21.3% (db). The resulting increase in protein content is likely due to the removal of residual sugars still present in the fermented biomass. However, it must be cautioned that using a decanter centrifuge on smaller volumes of fermented biomass usually results in reductions in overall yield due to losses on the larger surfaces of decanter centrifuges that are not minimized after scale up to large volumes.

Analyses of the sugar content of SH-*T. reesei* fermenting biomass slurry revealed an increase in its content of simple (fructose, glucose, sucrose) sugars during the fermentation process (Table 3). After initial centrifugation, the pelleted solids contained less raffinose, glucose and fructose while centrifugation significantly increased its sucrose content (Table 3 bottom – Final Solids).

Table 2. 150 L fermentation parameters using sorghum hominy from ADM and *T. reesei*.

Meal Lot Protein (db)	11.46%
Meal added (as is)	13.1 kg
Fresh Water Added	112 L
Total Slurry Volume	125 L
Cook Temp	121°C
Inoculum Amount	1 L (0.8%)
Incubation Temp	30°C
Agitation Rate	415 rpm
Aeration Rate	0.8 V/V/min

Table 3. Sugar profile of the 150 L slurry during fermentation (0-72 hrs) and after the initial decanter centrifuge separation step (before washing). All values given in g/L. N.D. is used to denote tested but not detected via HPLC Sugar-Pak column.

Incubation Time (hr)	Stachyose	Raffinose	Sucrose	Glucose	Fructose	Sum of Sugars
Raw SH	0.05	0.06	0.08	1.48	0.96	2.63
24	N.D.	0.97	0.58	1.65	0.16	3.37
48	N.D.	1.33	0.94	9.41	0.18	11.85
72	N.D.	1.34	1.19	13.77	0.41	16.71
Final Solids	N.D.	0.66	2.48	2.55	0.19	5.98

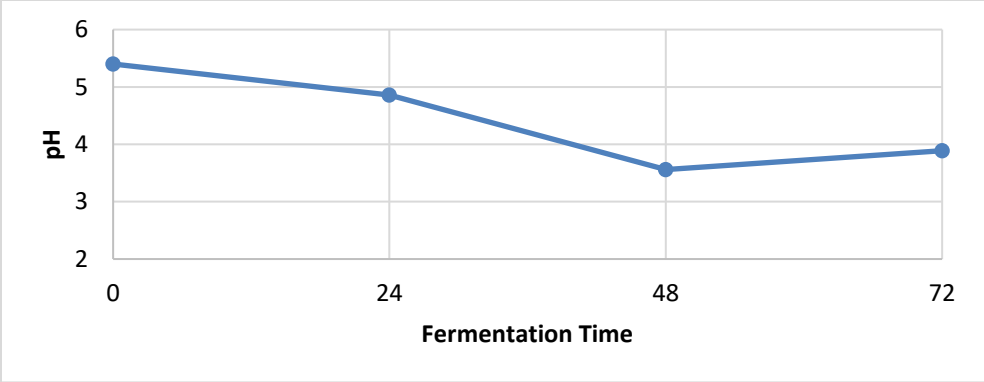

Figure 10. Slurry pH of the 150L sorghum hominy and *T. reesei* over the incubation time (72 h).

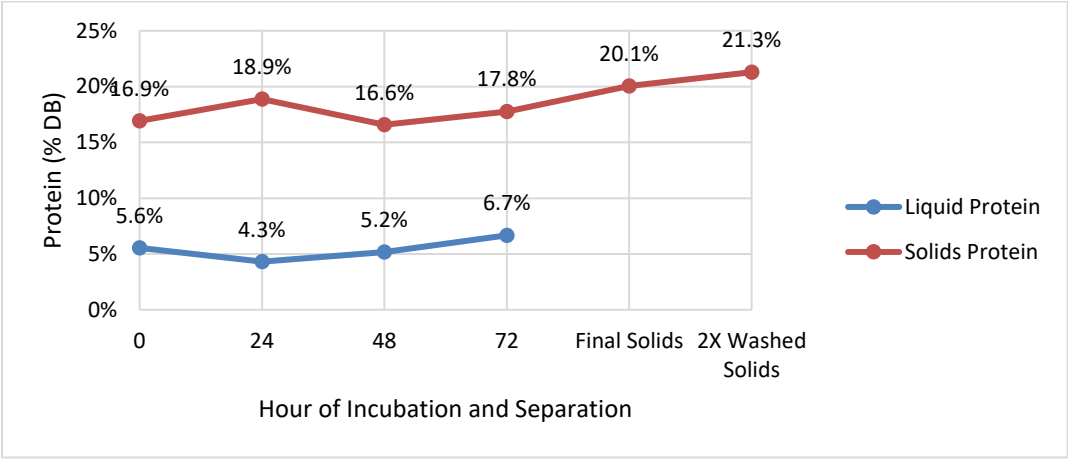

Figure 11. Protein concentrations (% db) of solids and centrate (liquid) fractions in sorghum hominy-*T. reesei* biomass during fermentation under submerged 150 liter conditions. After 72 hr., the resulting solids were harvested via centrifugation and subjected to washing with water. See text for details.

The proximate composition of the final SH-T. reesei bioprocessed product after fermentation in the 150 L fermenter and initial centrifugation without washing as compared to starting SH feedstock is shown in Table 4. These data reveal significant increases in the protein, crude fiber and ash content as well as the product's content of various minerals tested with the exception of potassium and magnesium. The amino acid content of the *T. reesei* fermented SH product also differed from its corresponding SH feed stock whereby all amino acids were increased due to increase in protein content of the final product. **Of interest are ~50% increases in key amino acids for feed ingredients in aquafeeds that include methionine, lysine and threonine as well as a two-fold increase in arginine.**

Table 4. Comparison of the chemical composition of standard sorghum hominy obtained from ADH and used for feedstock and the 150 L *T. reesei* fermented sorghum hominy. All determinations were performed by third party analysis.

Analysis	Unit	Sorghum Hominy	Fermented Batch -214 Sorghum Hominy		Sorghum Hominy	Fermented Batch -214 Sorghum Hominy
Proximates		as is			dry matter	
Moisture	%(m/m)	11.73	5.76		13.29	6.11
Dry matter	%(m/m)	88.27	94.24		100.00	100.00
Crude Fiber	%(m/m)	6.7	15.2		7.6	16.1
Crude Ash	%(m/m)	3.59	4.78		4.07	5.07
NDF	%	22.02			24.95	
Crude Fat	%(m/m)	8.4	12.4		9.6	13.2
Crude Protein	%(m/m)	11.4	20.4		12.9	21.6
ADF	%(m/m)	9.72			11.01	
Minerals						
Phosphorus	%(m/m)	0.62	0.95		0.70	1.01
Potassium	%(m/m)	0.8	0.46		0.91	0.49
Calcium	%(m/m)	0.05	0.1		0.06	0.11
Magnesium	%(m/m)	0.32	0.26		0.36	0.28
Zinc	PPM	37.6	50.8		42.60	53.90
Manganese	PPM	38.44	45.2		43.55	47.96
Copper	PPM	6.65	11.6		7.53	12.31
Iron	PPM	94.16	199		106.67	211.16
Sodium	%(m/m)	N.D.	0.06			0.06
Sulfur	%(m/m)	0.12	0.23		0.14	0.24
Amino Acids						
Alanine	%	0.87	1.28		0.99	1.36
Arginine	%	0.65	1.3		0.74	1.38
Aspartic Acid	%	0.89	1.18		1.01	1.25
Cystine	%	0.37	0.48		0.42	0.51
Glutamic Acid	%	1.76	2.76		1.99	2.93
Glycine	%	0.48	0.71		0.54	0.75
Histidine	%	0.27	0.46		0.31	0.49
Isoleucine	%	0.4	0.95		0.45	1.01
Leucine	%	1.06	2.19		1.20	2.32
Lysine	%	0.39	0.58		0.44	0.62
Methionine	%	0.19	0.28		0.22	0.30
Phenylalanine	%	0.48	0.82		0.54	0.87
Proline	%	0.65	1.13		0.74	1.20
Serine	%	0.49	0.7		0.56	0.74

Threonine	%	0.41	0.69		0.46	0.73
Tryptophan	%	0.15	0.19		0.17	0.20
Tyrosine	%	0.41	0.68		0.46	0.72
Valine	%	0.57	0.86		0.65	0.91
Total AA	%	10.5	17.2		11.9	18.3

5.2 OBJECTIVE 2: Evaluate SH and the bioprocessed SH as a feed ingredients in aquafeeds while determining their relative value in comparison to other feeds.

Evaluation of the performance of *T. reesei* bioprocessed SH as an aquafeeds ingredient vs. untreated SH and a standard commercial feed was performed using as described below. All fish rearing and growth studies were performed in Prairie AquaTech's aquatic laboratory except for histology evaluation that was completed in the company's second laboratory in Portland, ME. Tilapia fingerlings were sourced from commercial providers (Aquasafra www.tilapiaseed.com) stocked into holding tanks and fed a commercial tilapia diet (Zeigler www.zeiglerfeed.com) until fish were graded prior to initiation of feeding trial. A total of 5 replicates per diet were tested where all fish shared the same rearing water that was filtered and cleaned using standard biofiltration methods. Water quality was monitored daily and all mortalities were removed immediately. Fish were fed by hand 3 times per day where attempts to feed to true satiation was accomplished by multiple feed applications for a single "feed" until feed pellets were not consumed. Daily feed consumption was monitored on a daily/weekly basis where feed was increased on a weekly basis based on corresponding increases in biomass. Water temperature maintained at 26^{o}C (78^{o}F) and under continuous lighting conditions. Fish were weighed both a group basis at regular intervals and on an individual basis to obtain populational size distributions. Calculated values for feed conversion ratio (FCR), specific growth rate (SGR) and statistical methods were performed using standard methods.

Floating fish feeds were custom manufactured using an in house 3.25 inch Extru-Tech extruder. Feeds were formulated using an in house aquafeed formulation software program. A total of four test feeds were formulated and produced for the first tank-based fish trial. These were a tilapia control diet, a similar diet containing high (25% w/w) and low (15% w/w) inclusion rates of SH as well as a diet containing *T. reseei* bioprocessed SH (produced in 150 liter reactor described above) at an inclusion rate of 20% w/w.

All dissections were performed on individual fish immediately after lethal exposure to MS-222. Selected organs were removed, weighed and portions fixed in buffered 10% formalin. Fixed tissues were then processed for sectioning and staining by graded series of solvents prior to embedding tissue in paraffin and sectioning. Sections were attached to glass slides, rehydrated and stained using standard staining protocols. Sections were examined using either light or fluorescent microscopy and representative fields of view photographed.

Organoleptic panel testing of fillets was performed by groups of ten volunteers in a blinded manner where each was presented with a microwave cooked fillet sample and requested to evaluate and compare its properties to other samples. Parameters that were requested for the volunteers to analyze included ranking of fillets sampled based on texture, taste, aroma, moist/dry and after-

taste. Individual written responses were then collated and the average scoring determined using standard methods.

A total of four different pelleted feeds were manufactured after their custom formulation. Analyses of these feeds are shown in Table 5. All diets were 100% floating.

Table 5: Analysis of Test Feeds for Sorghum Trial.

AS IS ANALYSIS

	Production	Sorghum Low	Sorghum High	Fermented Sorghum (Batch 214)
	T.88.1	T.88.4	T.88.5	T.88.6
Moisture	5.26	5.70	5.00	4.18
Dry Matter	94.74	94.30	95.00	95.82
Protein (crude)	41.8	39.7	41.9	45.6
Fat (acid hydrolysis)	11.0	10.1	9.5	11.0
Fiber (crude)	4.02	3.82	2.36	4.65
Ash	7.31	7.88	7.88	7.64
Sulfur (total)	0.63	0.65	0.65	0.72
Phosphorus (total)	1.10	1.28	1.24	1.20
Potassium (total)	1.00	0.86	0.86	0.86
Magnesium (total)	0.28	0.26	0.23	0.24
Calcium (total)	1.24	1.60	1.66	1.40
Sodium (total)	0.18	0.18	0.20	0.24
Iron (total)	266	486	383	461
Manganese (total)	128	130	136	110
Copper (total)	58.80	58.70	58.20	51.90
Zinc (total)	398	399	398	350

Each of the test feeds were fed to replicate tanks of tilapia for an interval of 84 days (12 weeks). Average feed consumption for each of the test groups is shown in Table 6 and Figure 12. No palatability problems were observed where the tilapia receiving the *T. reesei* bioprocessed sorghum consumed the most feed as compared to the other 3 test groups.

Table 6: Comparison of Average Feed Consumption of Tilapia Fed One of Four Test Diets.

Average of Feed Consumed Cumulatively (gm)					
Trial Duration (Days)		21	42	63	84
Diet Type					
Standard Tilapia	Average	1555.2	2977.0	4054.8	4882.2
	S.D.	110.1	222.8	287.0	347.1
Sorghum Hominy (High)	Average	2187.6	2883.1	3999.7	4887.6
	S.D.	154.6	419.1	565.8	568.2
Sorghum Hominy (Low)	Average	1444.4	2879.5	3977.4	4896.7
	S.D.	119.9	219.9	315.4	401.7
Bioprocessed Sorghum (T. reesei)	Average	1866.0	3491.1	5103.2	6250.3
	S.D.	90.1	130.6	217.4	262.2

Table 7 and Figure 13 shows the average weights obtained by each of the groups after being reared on one of four test diets. Note that these average weights are similar with the exception of the

bioprocessed SH based diet that appeared to lag behind. However, due to the large variation in the average weights of trial fish tanks these differences were not statistically significant.

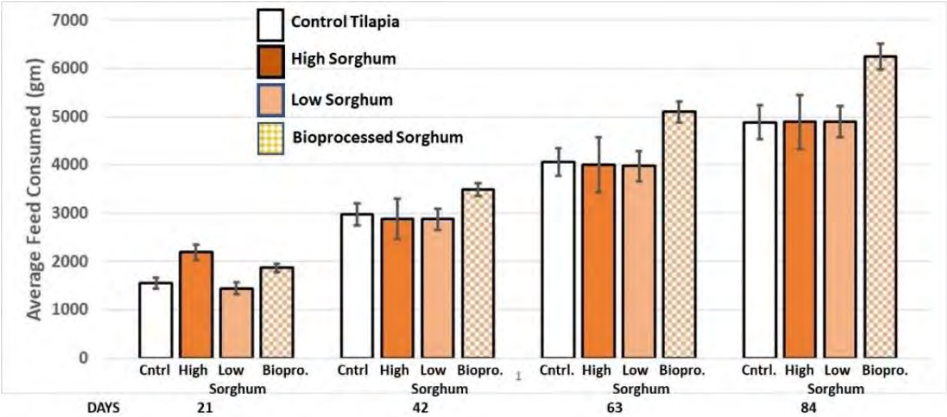

Figure 12: Comparison of the average feed consumption of each of the four test groups of tilapia.

Table 7: Comparison of Average Body Weights Obtained by Tilapia Fed One of Four Different Test Diets.

Average Body Weight (gm)						
Trial Duration (Days)		0	21	42	63	84
Diet Type						
Standard Tilapia	Average	242.9	346.4	431.9	473.8	506.8
	S.D.	8.8	22.4	33.8	41.9	53.3
Sorghum Hominy (High)	Average	245.5	346.4	440.6	489.0	523.0
	S.D.	5.8	19.8	51.2	72.2	86.3
Sorghum Hominy (Low)	Average	242.0	341.0	440.6	466.4	504.8
	S.D.	4.8	15.3	29.0	44.7	48.0
Bioprocessed Sorghum	Average	241.6	329.2	393.3	427.6	450.4
(T. reesei)	S.D.	4.4	22.5	55.6	73.5	92.3

Table 8 and Figure 14 display the corresponding specific growth rates for test tilapia shown in Table 7 and Figure 13. While SH fed tilapia grew well on feed containing SH at high and low inclusion rates, significant variation in individual weights of fish within this test cohort for the bioprocessed SH was observed (See Figure 15).

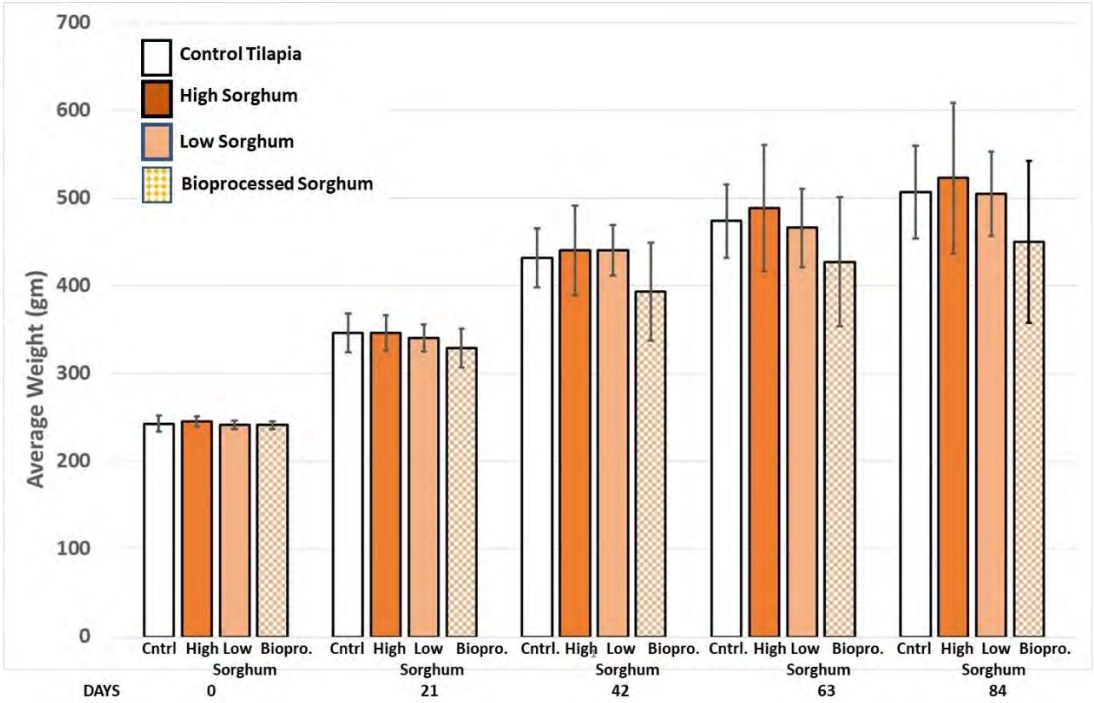

Figure 13: Comparison of average weights of test groups of tilapia fed one of four different test diets. See text for details.

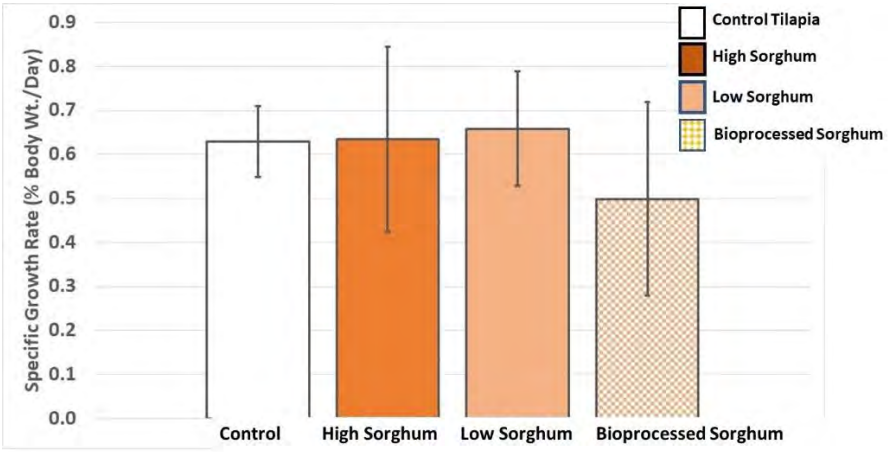

Figure 14: Comparison of specific growth rates for tilapia trial fish. See text for details.

A range of average weights were observed in trial test fish tanks that resulted in significant challenges in our efforts to statistically analyzed data between test groups. Figure 15 shows that test groups of tilapia grew at different individual rates during the 84 day trial and thus produced a significant range of individual weights that, in turn, produced variations in tank-based average weight measurements. These increases in individual size variation in test group fish is evident upon comparison of the weight distributions of test tilapia at trial initiation (Time 0) vs. the distribution of weights after 84 days of growth on each test diet (Figure 15).

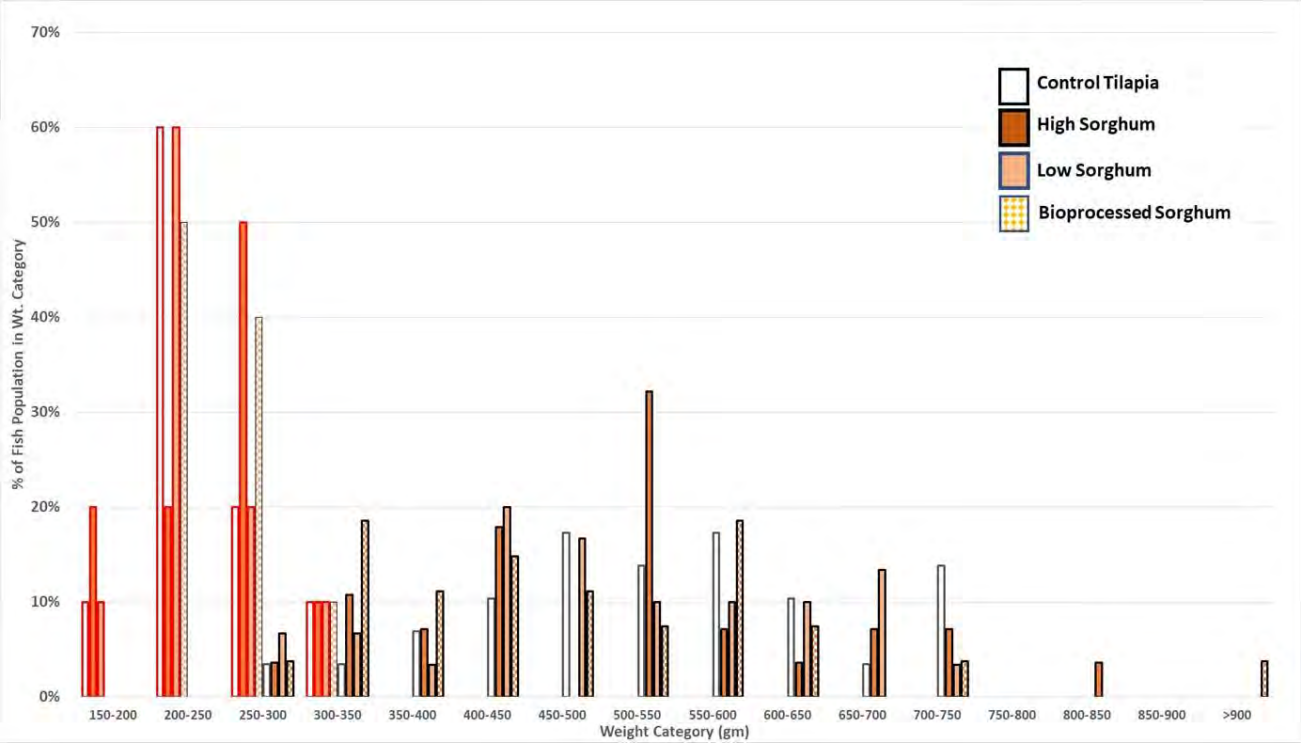

Figure 15: Comparison of the distribution of individual fish weights for the four test tilapia groups at time 0 (columns with red borders) vs. after 84 days of rearing on individual test diets (columns with black borders). Note that the variation in weights within all groups increased in conjunction with an increase in their average weight. See text for details.

Table 8: Comparison of Specific Growth Rates (SGRs) for Test Tilapia Groups

Average Specific Growth Rate (SGR)		
Trial Duration (Days)		84
Diet Type		
Standard Tilapia	Average	0.63
	S.D.	0.08
Sorghum Hominy (High)	Average	0.63
	S.D.	0.21
Sorghum Hominy (Low)	Average	0.66
	S.D.	0.13
Bioprocessed Sorghum (T. reesei)	Average	0.50
	S.D.	0.22

Table 9 and Figure 16 compare the feed conversion ratios (FCRs) displayed by tilapia fed one of four different test diets. **Note that the bioprocessed SH showed a significantly larger FCR as compared to any of the other three test groups.**

Table 9: Comparison of Feed Conversion Ratios for Tilapia Test Fish.

Feed Conversion Rate (FCR)					
Trial Duration (Days)		21	42	63	84
Diet Type					
Standard Tilapia	Average	1.49	1.57	1.76	1.88
	S.D.	0.12	0.12	0.19	0.21
Sorghum Hominy (High)	Average	1.44	1.57	1.80	2.01
	S.D.	0.22	0.35	0.57	0.66
Sorghum Hominy (Low)	Average	1.53	1.63	1.82	1.90
	S.D.	0.10	0.12	0.20	0.18
Bioprocessed Sorghum	Average	2.23	2.58	3.16	3.46
(T. reesei)	S.D.	0.50	0.82	1.03	1.12

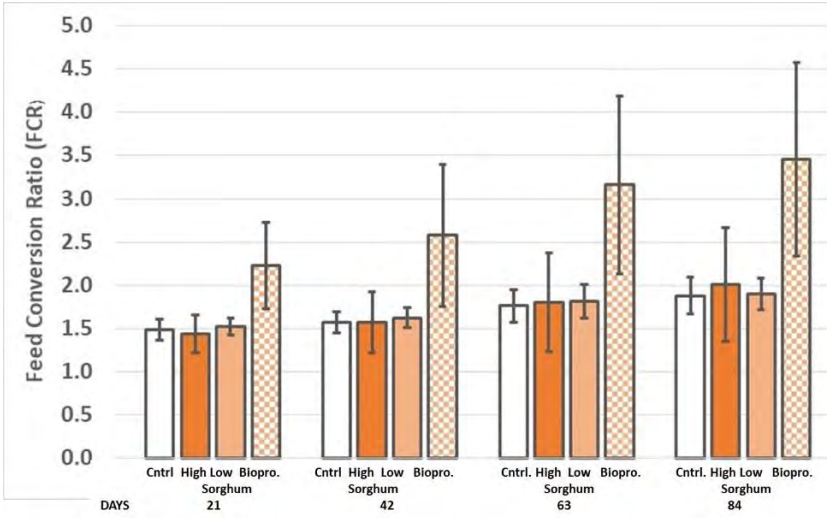

Figure 16: Comparison of feed conversion ratios of tilapia fed one of four test diets. See text for details.

Upon trial completion, test fish had achieved commercial market size and were tested for fillet yields as well as Hunter color analyses to determine if sorghum inclusion as a dietary feed ingredient altered the coloration of fillets. Tables 10 and 11 show the results of these analyses. The average fillet from each of the test groups was not different in either yield or coloration.

Table 10: Comparison of Fillet Yields of Test Tilapia

Fillet Yield (% of Body Weight)		
Tilapia Control	Average	25.6%
	S.D	1.3%
Sorghum (High)	Average	25.9%
	S.D	1.6%
Sorghum (Low)	Average	27.0%
	S.D	1.6%
BioProcessed Sorghum	Average	28.1%
	S.D	1.9%

Table 11: Hunter Scan Color Analysis of Fillet Coloration of Tilapia Test Fish.

HUNTER SCAN PARAMETER		L (Lightness)	A (Red-Green)	B (Yellow-Blue)
Diet Type				
Tilapia Control	Average	39.7	-2.85	4.92
	S.D.	1.09	0.48	0.79
Sorghum (High)	Average	40.32	-2.84	4.87
	S.D.	0.75	0.34	1.16
Sorghum (Low)	Average	39.97	-2.68	4.89
	S.D.	2.03	0.4	1.01
Bioprocessed Sorghum	Average	40.39	-2.85	4.78
	S.D.	1.34	0.48	1.2

L - Lightness - 0 = black; 100 = white
A - Red = Positive; Green = Negative
B - Yellow = Positive; Blue = Negative

Fillets were also subjected to organoleptic or sensory taste testing as described previously. A total of 8 participants evaluated the samples. These sensory testers reported that fillets from fish reared on test feeds containing a high and low SH content were considered "good tasting" as compared to fillets from tilapia fed the control diet that were sampled at the same time. In these evaluations, the taste evaluators commented that the fillets from SH reared tilapia had little flavor and displayed a bland taste that is preferred by seafood customers**. By contrast, the fillets derived from fish reared on the bioprocessed SH diet were evaluated as "unacceptable and bad tasting" by all subjects due to the presence of an "off" flavor that was described as a sharp metallic taste to the fillet.**

A subset of fish from each of the tilapia test groups was further analyzed to determine if changes in biological parameters used as metrics in the performance of farmed fish were present. A total of 5 fish from each test group were selected and dissected to determine various organ weight to body weight ratios as shown in Table 12.

Table 12: Comparison of Liver (HepSIndex), Spleen (SplenSIndex) and Visceral Fat (ViscFatIndex) Weights to Body Weights in Test Tilapia Fish After 84 Days of Test Diets.

Biological Parameters		Wt.	K	HepSIndex	SplenSIndex	ViscFatIndex
Tilapia Control	Average	529.51	2.18	1.80	0.072	4.76
	S.D	56.85	0.22	0.42	0.015	0.25
Sorghum (High)	Average	532.58	2.27	1.76	0.076	5.93
	S.D	38.34	0.06	0.33	0.023	1.76
Sorghum (Low)	Average	450.35	2.10	2.19	0.068	5.14
	S.D	62.17	0.26	0.78	0.020	1.24
BioProcessed Sorghum	Average	448.44	2.11	1.92	0.084	3.83
	S.D	99.65	0.21	0.65	0.014	1.47

No significant differences were observed in these parameters for any of the test groups as compared to the control group. To further evaluate possible differences between test groups,

histological analyses of these organs were performed. Figure 17 shows the appearance of liver tissue containing varying degrees of liver vacuolization. The appearance of whitish inclusion bodies within liver cells has been shown to be composed of lipid and glycogen and abnormalities in the liver storage of these important energy and growth building blocks correlated with problems in the overall metabolic profile of fish being fed feeds that are nutritionally imbalanced or contain compounds deleterious to fish. As noted by the arrows, fish liver tissue can display varying degrees of vacuolization from low (Panel A) to high (Panel C). Such liver vacuolization can be quantified by assessing both the frequency and size of liver cell vacuoles so as to place them into either low (A), medium (B) or high (C) vacuolization categories. Such categories are then assigned a 1-3 numerical score than can be averaged and compared to date from other fish.

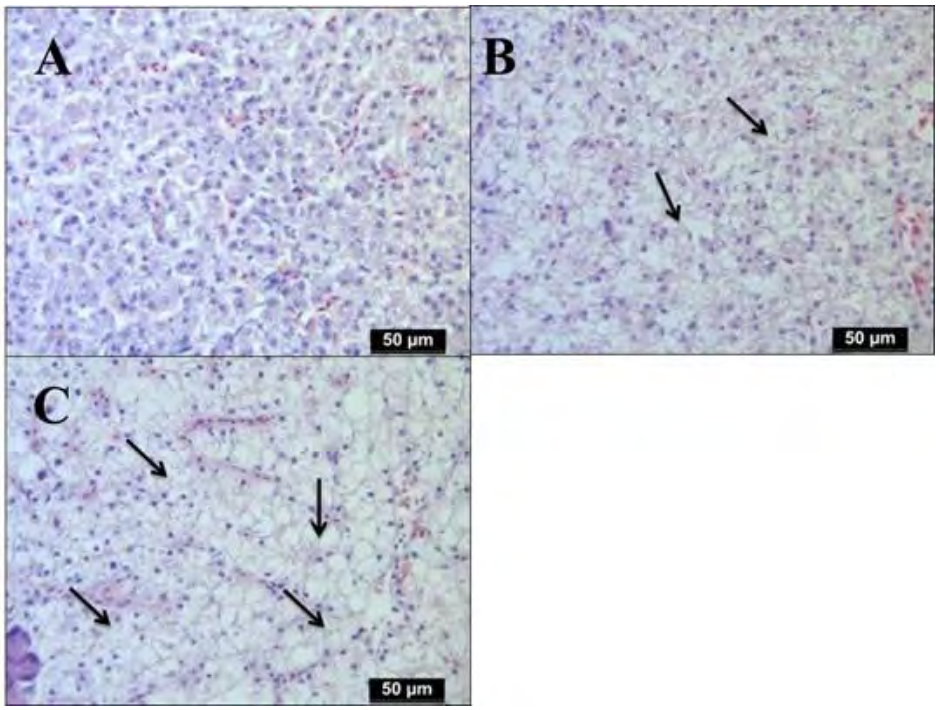

Figure 17: The histological appearance of varying degrees of vacuolization of liver tissue ranging from low (Panel A) to medium (Panel B) to high (Panel C) as shown by arrows denoting the appearance of individual liver vacuoles within liver tissue. See text for details.

Figure 18 shows data comparing the degrees of liver vacuolization present in tilapia fed one of three different test diets as compared to control. These data show that tilapia fed either of the three sorghum-containing test diets displayed a similar degree of liver vacuolization.

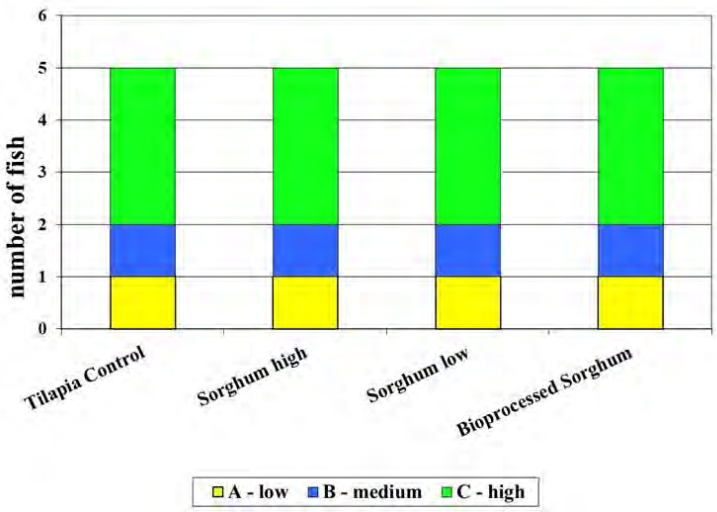

Figure 18: Comparison of degrees of liver vacuolization present in trial tilapia. See text for details.

Figure 19 shows a representative photomicrograph of spleen tissue from test tilapia. We have noted previously that commercial tilapia fingerlings possess granulomas in their splenic tissue (personal communication). Thus, the presence of granulomas in the spleens of these trial fish should be regarded as an incidental finding.

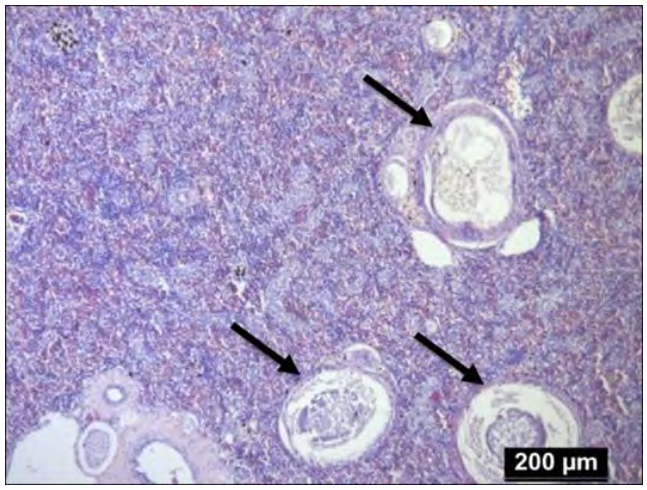

Figure 19: Representative photomicrograph of spleen tissue from tilapia trial fish. The three arrows denote the appearance of granulomas commonly present in the spleens of tilapia sold commercially as fingerlings. See text for details.

By contrast, staining of splenic tissue using a Prussian Blue staining protocol results in the highlighting of regions of tissue that contain the iron rich protein hemosiderin as bluish stained tissue as shown in Figure 20. These hemosiderin-rich regions can be associated with specific areas of splenic tissue associated with the turnover of macrophages often containing the pigment melanin (named melanomacrophage centers or MMCs) or located within the white and red pulp of the splenic tissue itself. Examples of both of these staining patterns are shown in Figure 20. The presence of increased hemosiderin is associated with increases in tissue iron content that have been shown to predispose fish to a variety of iron-dependent bacterial pathogens. Thus, diets that results

in the increased deposition of tissue iron are not desirable and the dietary content of iron in feed formulations should be adjusted accordingly.

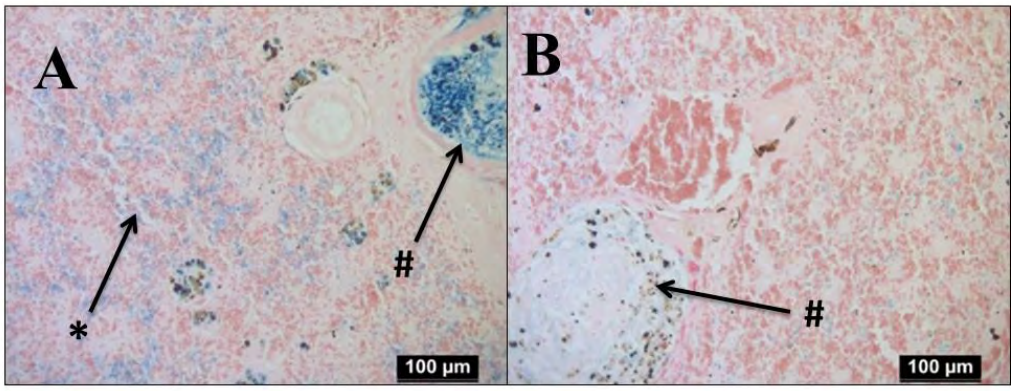

Figure 20: Staining of splenic tissue using Prussian Blue staining method. (Panel A) – The arrows denote the appearance of bluish-colored hemosiderin rich areas of tissue located within an MMC (pound sign) or dispersed in the white and red pulp of splenic tissue (asterisk). (Panel B) – The arrow with pound sign denotes the appearance of an MMC containing both hemosiderin as well as darker melanin granules. See text for details.

Figure 21 provides semiquantitative measurements of the presence of bluish-staining hemosiderin present in trial fish where hemosiderin is located within MMCs. Note that test fish receiving SH-based diets display an increase in MMC associated hemosiderin staining particularly those fish reared on bioprocessed SH.

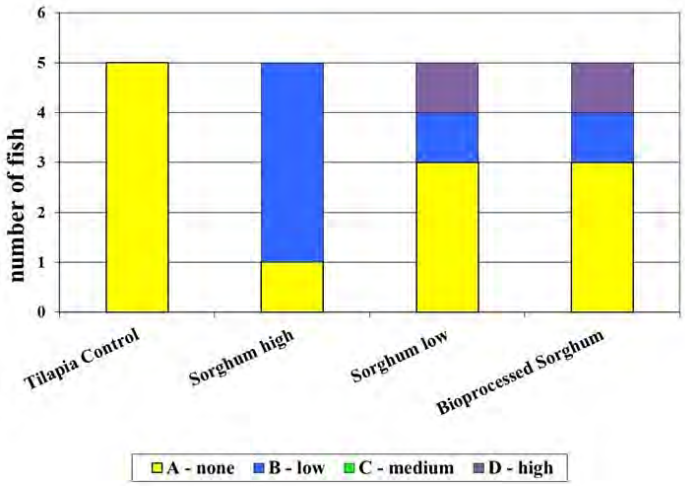

Figure 21: Comparison of hemosiderin deposition with melanomacrophage centers (MMCs) in splenic tissue of tilapia fed one of three test diets. See text for details.

Similarly, Figure 22 shows data on hemosiderin staining of splenic tissue using the same methodology as described for Figure 10. These data also show an increase in tissue iron deposition present in tilapia fed SH test diets. Further work is necessary to understand the mechanism(s) underlying these findings since dietary iron content of sorghum test diets were 40-80% greater as compared to control (See Table 5).

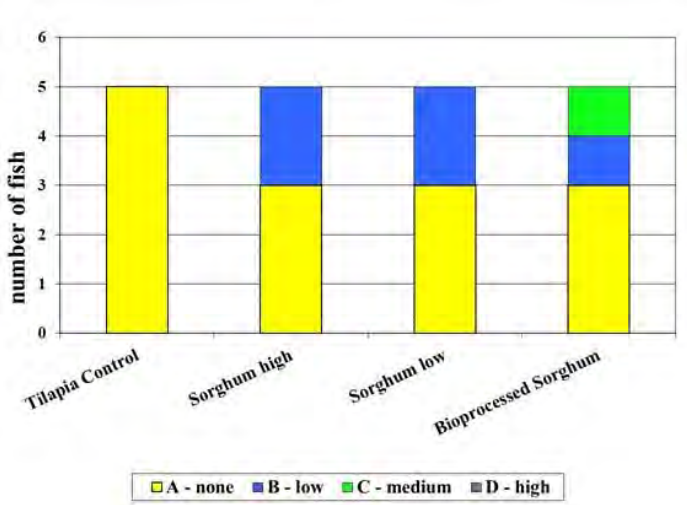

Figure 22: Comparison of hemosiderin deposition in splenic tissue pulp of tilapia fed one of three test diets. See text for details.

Figure 23 shows a representative photomicrograph of kidney tissue from a tilapia fed one of three test diets. Careful examination of kidney tissue sections from all three test groups of fish revealed no significant differences in morphology or staining.

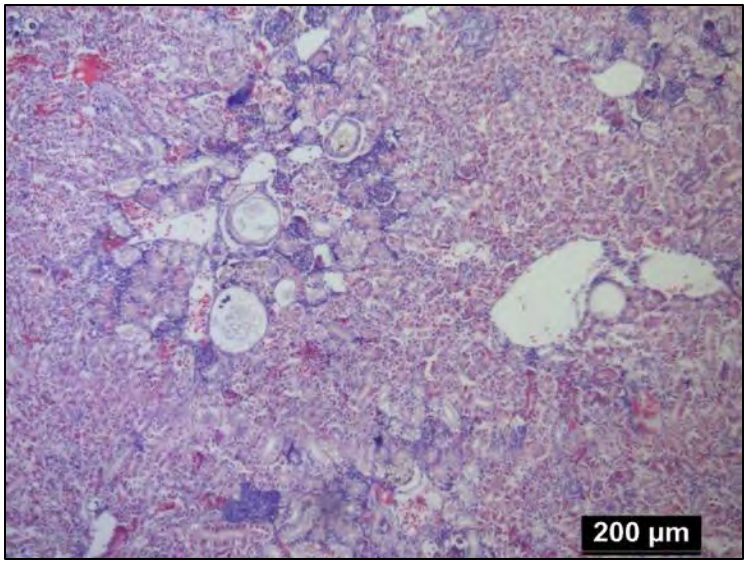

Figure 23: Representative photomicrograph of kidney tissue from tilapia fed one of three test diets as described in the text.

Figure 24 shows representative photomicrographs of intestinal tissue from a tilapia fed one of three test diets in the trial described above. After careful examination, we observed no significant differences in either the proximal, mid or distal intestinal segments of either Control or sorghum-containing test diets.

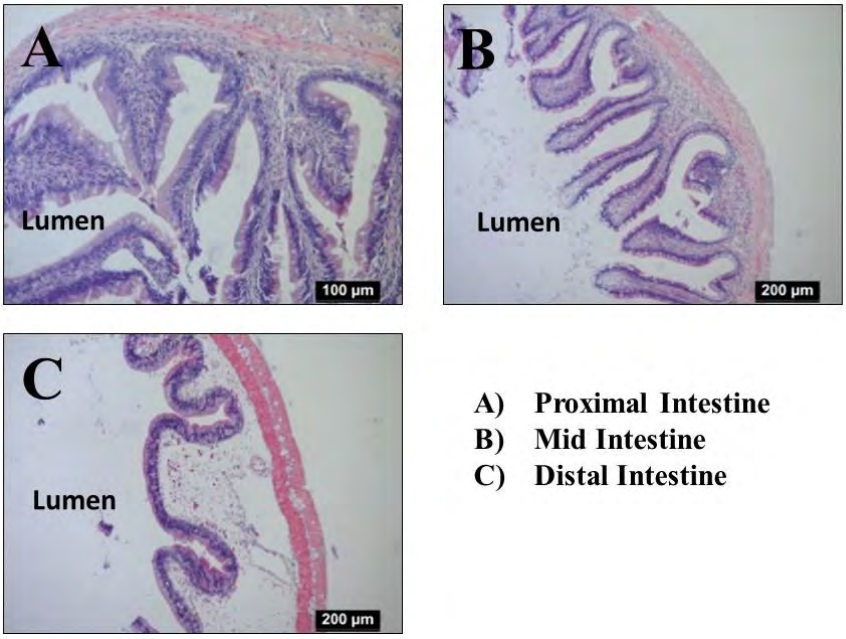

Figure 24: Representative photomicrographs of regions of intestine from tilapia fed one of three test diets. See text for details. The lumen of the intestine lined with its absorptive surface of epithelial cells is indicated by labeling. See text for details.

Table 13 shows the average blood values for tilapia fed one of three test diets in the trial described above. No significant differences in hematocrit were observed for any of the four test groups.

Table 13: Comparison of hematocrit values for tilapia test fish.

Biological Parameter	Hematocrit (% Blood Volume)	
Tilapia Control (n=33)	Average	37.52
	S.D	4.38
Sorghum (High) (n=32)	Average	37.38
	S.D	3.54
Sorghum (Low) (n=33)	Average	34.82
	S.D	4.65
BioProcessed Sorghum (n=32)	Average	34.81
	S.D	4.17

In summary, data obtained from the 84 day feeding and growth trial with test groups of tilapia as summarized above support the following conclusions regarding SH as well as SH bioprocessed with *T. reesei* selected on the basis of its abilities to bioprocess SH constituents. These are:

- **Both SH and *T. reesei* bioprocessed SH can be incorporated into extruded aquafeeds with good outcomes regarding pellet quality and performance.**
- **SH and bioprocessed SH feeds are highly palatable to tilapia and support their growth under standard tank-based conditions.**

- **The growth and feed efficiency of tilapia fed SH containing tilapia feeds is similar to that achieved by standard soy and poultry meal based commercial tilapia diets.**
- **The growth of tilapia fed *T. reesei* bioprocessed is similar to control fed tilapia and may be reduced (unable to determine based on large amount of size variation in fish). However, these data did demonstrate a significant reduction in feed efficiency in tilapia fed *T. reesei* bioprocessed SH.**
- **The spleen tissue of tilapia fed *T. reesei* bioprocessed SH shows evidence of increase tissue iron deposition.**
- **Although the fillet yield and coloration of test tilapia fed T. reesei bioprocessed SH containing feed are similar to control and other SH test groups, these fillets display a strong off flavor that is rejected by a human taste test panel.**

Due to these findings described in Tables 5-13 and Figures 12-24 obtained in trial of sorghum tilapia test diets for 84 days duration, a second pilot scale trial consisting of 4 tanks (200 fish/tank) and 800 tilapia was performed that focused on a possible combination of SH and a bioprocessed soy product called ME-PRO® that is produced by our company, Prairie AquaTech (www.prairieaquatech.com) . ME-PRO® is bioprocessed from washed soybean meal using aerobic fermentation with a yeast-like organism *A. pullulans* (used for studies on SH as described above).

The central goal of this second pilot scale trial of 21 days was two-fold. The first goal was to determine if the combination of SH and ME-PRO may confer a growth benefit for fish reared on such test diets. The second goal was to determine if SH might be utilized as a feed ingredient for its mechano-elastic properties enabling feed manufacturers to produce SH-containing feeds with either improved pellet characteristics or dramatic reductions in capital costs in feed production equipment.

As an initial step in SH feed development, SH was run through an Extru-Tech e325 extruder at various inclusion rates to determine its ability to form a stable floating aquafeed. Surprisingly, floating aquafeed pellets could be formed with 100% SH to replace both whole wheat and wheat middlings (mids). Using this knowledge, a simple formulation was designed by combining a high protein soy product (ME-PRO with 70% protein) together with SH to produce a suitable tilapia diet. As a second step, based on the relative ease by which the combination of SH + ME-PRO forms floating extruded pellets, a pellet press method using a combination of a Hobart 4146 meat grinder modified with a die plate and cutting wheel to produce feed pellets was designed and tested. In order to maximize this low cost simple system, a small propane camping stove was employed to supply additional heat at the die plate. Oil was applied to the dried feed pellets using a mixing bowl and cement mixer for small and larger batches respectively. While this screw pressed aquafeed was denser than its extruded counterpart and did not float on water, it did display the ability to retain its integrity for an interval that was long enough to be used as a product for tilapia fish production.

Figure 25 as well as Tables 14 and 15 summarize the characteristics of the 3 test SH-containing feeds and corresponding extruded control feed manufactured for the second trial. Extruded test diets containing the combination of SH and ME-PRO displayed excellent characteristics. As expected the screw pressed diet containing a combination of SH and ME-PRO was significantly

denser and did not float on water. The rationale for testing this diet is that it can be manufactured with only a small amount of capital investment (<$15,000) and thus can be used by fish farmers where access to either expensive feed mills or extruded feeds are unaffordable or undesirable (see economic analysis section below).

Table 14: Comparison of Bulk Density and Flotation Characteristics of Tilapia Test Diets.

FEED ANALYSIS PARAMETERS		
Diet Type	Bulk Density gm/cm3	Float Test % Floating/20min.
Standard Tilapia	0.37	100
Extruded Sorghum High ME-PRO	0.34	100
Extruded Sorghum Low ME-PRO	0.34	100
Screw Pressed Sorghum Low ME-PRO	0.51	1
	n=10	n=10

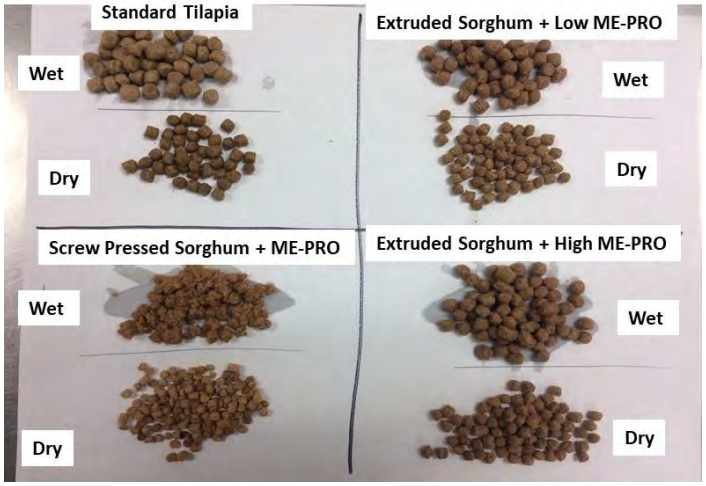

Figure 25: Appearance of control and test trial diets after manufacture (Dry) and after an exposure to water for 20 minutes. All diets performed well with the exception of the screw pressed SH diet that did not float but retained its integrity in water. See text for details.

Test tilapia consumed all four test diets without palatability issues during the trial of 21 days (Figure 26). However, consumption of the screw pressed test feed was higher than other experimental feeds tested. As expected, water quality parameters for tanks containing fish receiving screw press test feed were reduced (higher suspended solids) as compared to tanks receiving extruded feeds. No adverse effects on fish were observed during the trial.

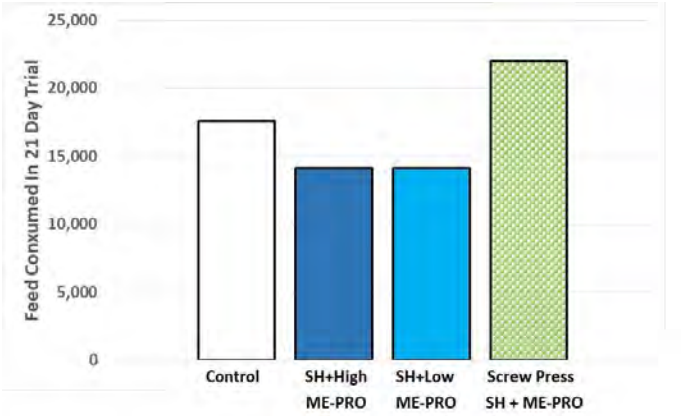

Figure 26: Comparison of feed consumption for four test tilapia fish for trial duration of 21 days. See text for details.

Table 15: Analysis of Tilapia Test Feeds for Second Fish Trial.

AS IS ANALYSIS		5mm feeds		
	Standard Tilapia Production Diet	Extruded Sorghum + Low level ME-PRO	Screw-pressed Sorghum + Low level ME-PRO	Extruded Sorghum + High level ME-PRO
	T.118.1	**T.118.2**	**T.118.3**	**T.118.4**
Moisture	7.47	8.91	13.98	9.15
Dry Matter	92.53	91.09	86.02	90.85
Protein (crude)	41.1	40	37.3	43.9
Fat (crude)	8.16	8.69	10.6	6.98
Fiber (crude)	3.27	4.48	3.92	4.23
Ash	7.47	5.39	5.06	4.73
Sulfur (total)	0.6	0.45	0.42	0.53
Phosphorus (total)	1.2	0.89	0.8	0.82
Potassium (total)	1	0.62	0.57	0.54
Magnesium (total)	0.27	0.27	0.26	0.24
Calcium (total)	1.47	0.79	0.48	0.59
Sodium (total)	0.16	0.19	0.17	0.16
Iron (total)	366	187	150	190
Manganese (total)	151	94.1	87.8	84.8
Copper (total)	66.4	83.9	83.2	77.1
Zinc (total)	390	345	326	318
Aspartic acid	3.08	3.84	3.65	4.08
Threonine	1.18	1.4	1.33	1.44
Serine	2.02	1.96	1.78	2.02
Glutamic acid	5.87	6.16	5.78	6.7
Proline	2.57	1.98	1.78	2.05
Glycine	2.66	1.65	1.47	1.63
Alanine	2.02	1.84	1.67	1.78
Cystine	0.72	0.58	1.03	0.6
Valine	1.96	1.9	1.74	1.8
Methionine	0.89	0.56	0.53	1
Isoleucine	1.57	1.76	1.63	1.73
Leucine	2.72	3.03	2.8	3.02
Tyrosine	1.18	1.52	1.38	1.57
Phenylalanine	1.7	1.92	1.85	2.03
Lysine (total)	2.79	3.66	3.35	3.78
Histidine	0.88	1.03	0.95	1.04
Arginine	2.56	2.7	2.53	2.73
Tryptophan	0.42	0.44	0.46	0.6
Pellet weight (g/pellet	0.047	0.038	0.033	0.033
Bulk Density (g/L)	370.5	338.2	513.2	341.1
Percent Floating	100%	100%	1%	100%

Figure 27 shows increases in average body weight for each of the test groups during the pilot trial. Note that both extruded SH diets produced growth in tilapia that appeared similar (SH+ high ME-PRO>SH + Low ME-PRO) and perhaps slightly less than fish reared on a standard tilapia control diet. Interestingly, fish reared on the screw pressed SH diet grew at a rate similar to that displayed by control fish. Similar data was obtained by measurements of individual fish weights to determine the size distribution of groups as shown in Figure 28.

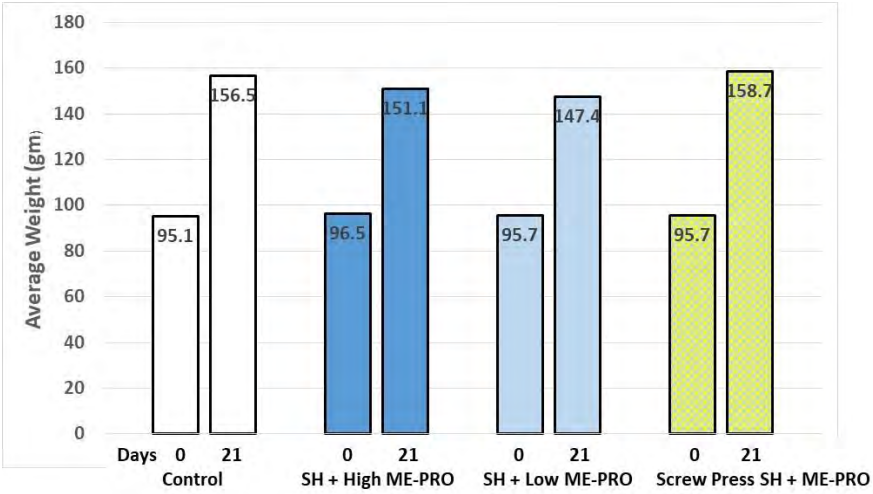

Figure 27: Comparison of average weight of four test tilapia fish groups at the start (Time 0) and end (Time 21) of the trial. See text for details.

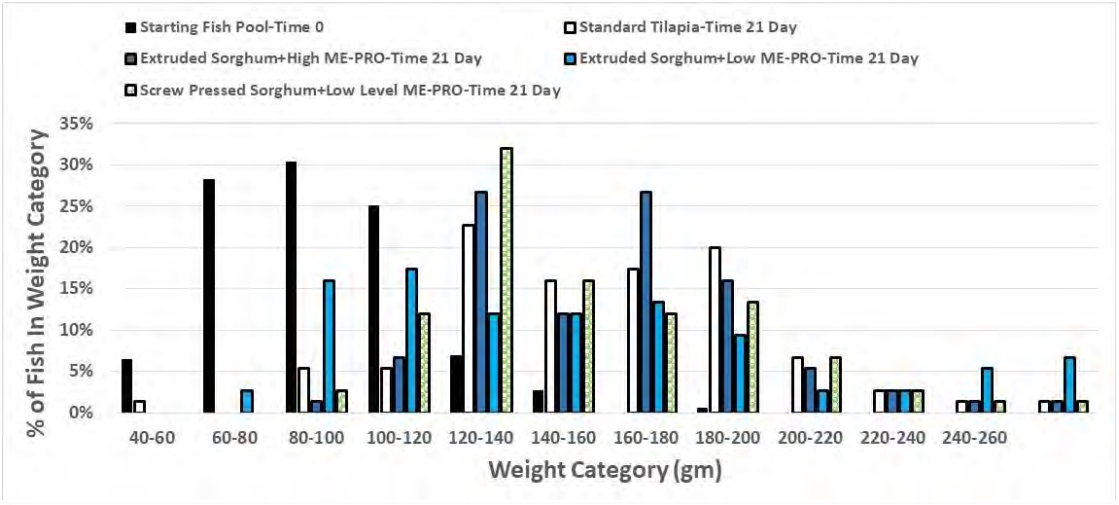

Figure 28: Comparison of distribution of individual weights for tilapia fed one of three SH containing diets as compared to control. See legend for column designations and text for details.

Feed conversion ratios for each of the four test groups are shown in Figure 29. These data are notable for the apparent lower FCRs displayed by SH containing extruded diets and an FCR that is comparable to control exhibited by fish reared on the SH-based screw press diet. Larger more extended studies as described for bioprocessed SH (see above) are necessary to further define these promising pilot scale results.

To determine whether any changes in body characteristics or selected organs occurred in tilapia reared in this pilot scale trial an initial survey was performed as shown in Table 16. No significant differences were observed in condition factor, liver, spleen or visceral fat weights in fish receiving these pilot scale test diets.

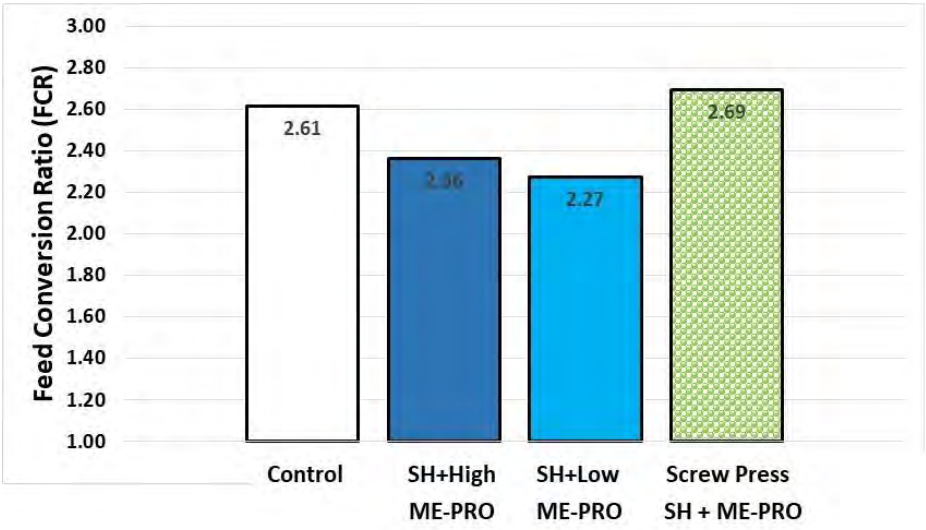

Figure 29: Comparison of feed conversion ratios (FCRs) for each of four test groups of tilapia. See text for details.

Table 16: Comparison of Tissue Weights vs. Body Weights for Tilapia Test Groups.

Biological Parameters		Wt.	K	HepSInde:	SplenSIndex
Tilapia Control	Average	152.3	2.3	3.2	0.06
	S.D	20.1	0.1	0.3	0.02
Sorghum + High ME-PRO	Average	136.2	2.2	3.2	0.06
	S.D	30.2	0.1	0.6	0.02
Sorghum + Low ME-PRO	Average	148.8	2.2	3.1	0.04
	S.D	16.7	0.0	0.6	0.01
Screw Pressed Sorghum + Low ME-PRO	Average	152.7	2.3	3.2	0.04
	S.D	54.5	0.2	0.7	0.01

In summary, data obtained from this pilot scale 21 day feeding and growth trial with test groups of tilapia as summarized above support the following conclusions regarding SH as a possible feed ingredient when formulated in conjunction with a novel bioprocessed soy ingredient called ME-PRO®. These are:

- **When combined with ME-PRO®, SH appears to be a promising replacement for whole wheat and wheat middlings in extruded aquafeeds that should be further developed based on the ability of SH to provide a high quality extruded pellet that yields good performance in tilapia.**
- **Use of SH together with ME-PRO can provide the ability to manufacture screw press tilapia feeds with good growth performance and acceptable pellet integrity for use in tilapia farming where high quality extruded feeds are either unavailable or too expensive for fish producers to utilize.**

5.3 OBJECTIVE 3: Estimate commercial scale production costs (CAPEX/OPEX), market size and value, return on investment (ROI) potential, and timeline for deployment

Based on the data summarized in **OBJECTIVES #1 and #2** of this report, we are now able to provide some preliminary estimate of commercial scale production costs for a sorghum hominy-*T. reesei* processed product. Such estimates are currently framed around two significantly different production formats. These are:

1. Submerged Liquid-based Fermentation in Large Stainless-Steel Fermentation Equipment- The parameters of this fermentation format are summarized in **OBJECTIVE #1**. While this liquid fermentation format produces reliable outcomes and has been the subject of most of our efforts, the "stickiness" of the SH-*T. reesei* fermenting biomass will likely require significant (costly) modifications to produce optimal and consistent results. Based on our present knowledge of costs associated with liquid state fermentation of feedstocks such as sorghum hominy under standard conditions (not taking into account of any mixing modifications, etc.), Table 17 outlines preliminary estimates of operating costs to perform SH bioprocessing using *T. reesei* as described in this report. Note that these cost estimates do not include CAPEX costs required to build any commercial scale plant or a standard depreciation of such CAPEX costs.

Table 17. Estimate of Possible Projected Process Cost Estimates for Bioprocessed SH at Commercial Scale (>5000 tons/yr.)

Item	Cost per ton of Bioprocessed SH
Sorghum Milo Hominy	$127
Water	$10
Sulfuric Acid	$20
Natural Gas	$91
Electric	$30
Organism	$9
Repairs and Maintenance	$16.8
Contingency	$13.7
Labor	$73
Total Cost	**$390.5/ton**

2. Solid State Fermentation in Lower Cost Plastic Tank or Troughs – The data shown in Figure 9 provides support for the possible use of a solid state fermentation platform for commercial production of a SH-*T. reesei* bioprocessed product. This new possible solid state product requires further characterization.

Based on our present knowledge, the data obtained from this work does not support the performance or financial viability of using aerobic fermentation as tested in this project to bioprocess sorghum hominy as a means of producing a value-added ingredient for aquafeeds. The cost estimates provide above for liquid submerged fermentation will place the OPEX of such an aerobic fermentation process for sorghum above the price points of more widely

available plant based feed ingredients such as commodity based soybeans (~$335/ton; 46% protein) or corn based DDGS ($110/ton; 22% protein). Submerged liquid aerobic fermentation requires large investments in stainless steel fermenters and sterile operational conditions. These items will require a very large CAPEX investment for such a plant (e.g. a 30,000 ton soybean meal processing plant utilizing the same aerobic fermentation platform requires $40 million USD in CAPEX investment).

Alternatively, these data summarized in Objective #2 provide significant support for the use of SH as a ready-made feed ingredient for extruded aquafeeds that could be utilized to compete with whole wheat and wheat middlings. The carbohydrate content of SH appears to be both gelatinized during the extrusion process to allow creation of a high-quality pellet that displays a growth and feed utilization performance that is similar to standard tilapia diets. The resulting fillet product appears acceptable to the seafood consumer based on both color and taste. It is recommended that additional technical validation work be performed together with efforts to make SH more available as a feed ingredient for extruded feed mills.

The combination of performance and financial metrics will also dictate possible uses of SH as a feed ingredient in extruded aquafeeds. Tilapia diets vary with respect to protein and lipid concentrations that vary quite a bit globally, depending upon the fish culture style (extensive, pond-based or intensive, tank-based). Typically, tilapia feeds utilized in more extensive culture situations (outdoor ponds and lagoons) often range between 32 and 35% crude protein and 4 to 6% crude fat since they rely on the primary productivity in the ponds to supplement the complete feed addition. For more intensive production systems, typical protein and lipid concentrations range from 36-41% crude protein and 6-10% crude fat levels in production diets. Accordingly, the costs of these diets can vary greatly as well, depending upon production site, production type, freight, ingredient quality etc., but generally range anywhere from $0.32/pound ($640/ton) to $0.52/pound ($1040/ton).

Table 18 provides an economic analysis of the possible costs and use of SH based extruded feeds for tilapia aquaculture. Note that for both extruded feeds containing high (25%) and low (15%) SH, competitive feed pricing can be obtained using already existing infrastructure and feed distribution networks. Interestingly, similar pricing and value could be obtained via the manufacture of SH containing feeds using a low CAPEX screw press method. Potential customers for possible lower quality screw press based feeds are likely to depend on pond or lagoon-based culture systems and therefore less particular about feed quality and its floatation. The ability to provide affordable screw press feeds for groups of farmers would represent a possible significant expansion of the tilapia feed market. Similar technologies could possibly be applied to manufacture of catfish feeds.

Table 18: Characteristics and Possible Pricing Matrix for High and Low Sorghum Extruded as well as Screw Press Feeds Containing Sorghum Hominy.

Diet	Protein/Lipid	$USD Price per pound Extruded	$USD Price per pound Screw-Press
High Sorghum	36/6	0.39	0.32
Low Sorghum	42/9	0.44	0.36

Prices include a 10% margin on final feed, off-specification ME-PRO® at $750/ton and manufacturing costs of approximately a 67% discount for the Screw-Press feeds. These feeds met all known nutritional requirements for optimal tilapia growth, but did not contain any wheat products (either whole wheat or wheat middlings) that are typically included in formulations primarily for their starch and/or filler content (i.e. pellet durability and integrity and floatability of the feeds).

Producers

It is anticipated that sorghum producers are also included as primary beneficiaries of this technology, as it will substantially increase the demand sorghum derivatives. The secondary target audience will include underserved areas of rural populations that have embraced fish farming but are limited in access to do so based on high prices of quality feeds. Lastly, another beneficiary of this technology development is anticipated to be aquaculture feed manufacturing companies that could utilize SH instead of wheat based products.

Return on Investment

All agricultural processors are interested in increasing the diversity and value of products they generate. An example of this commercial product diversification is the corn wet milling industry. By comparison, grain dry milling operations have not developed their base commodity into similar diverse product lines. Integration of our technology into sorghum dry milling operations will increase product diversity and value, providing a competitive advantage. Successful implementation of our technology to sorghum products will translate into higher demand for and price of sorghum, which will benefit sorghum producers.

APPENDICIES

None

LiST OF ABBREVIATIONS

Abbreviation	Meaning	Abbreviation	Meaning
RAS	Recirculating aquaculture system	COGS	Cost of goods sold
SMF/SH	Sorghum mill feed/sorghum hominy	min	Minutes
HP-SH	High protein sorghum hominy	h	Hours
MMT/yr	Million metric tons/yr	^{o}C	Degree Celsius
CAPEX	Capital equipment expense	mL	Milliliter

OPEX	Operating expense	L	Liter
ROI	Return on investment	GYE	Glucose yeast extract
DDGS	Distillers' dried grains with solubles	GM	Genetically modified
rpm	Revolutions per minute	db	Dry matter basis
g	Gram	kg	kilogram
TS	Total solids	TSS	Total suspended solids
TDS	Total dissolved solids		

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